HOKKAIDO UNIVERSITY

Title	Effect of Subcultivation of Human Bone Marrow Mesenchymal Stem on their Capacities for Chondrogenesis, Supporting Hematopoiesis, and Telomea Length
Author(s)	Nakahara, Masaki; Takagi, Mutsumi; Hattori, Takako; Wakitani, Shigeyuki; Yoshida, Toshiomi
Citation	Cytotechnology, 47(1-3), 19-27 https://doi.org/10.1007/s10616-005-3752-9
Issue Date	2005-01
Doc URL	http://hdl.handle.net/2115/933
Rights	The original publication is available at www.springerlink.com
Type	article (author version)
File Information	CT47(1-3).pdf

Instructions for use

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Effect of subcultivation of human bone marrow mesenchymal stem cells on their capacities for chondrogenesis, supporting hematopoiesis, and telomea length

Masaki Nakahara,[1] Mutsumi Takagi,[1*] Takako Hattori,[2] Shigeyuki Wakitani,[3] and Toshiomi Yoshida[1]

[1]International Center for Biotechnology, Osaka University, 2-1 Yamada-oka, Suita, Osaka 565-0871, Japan, [2]Department of Orthopedic Surgery, Osaka-Minami National Hospital, 2-1 Kidohigashi-machi, Kawachinagano, Osaka 586-8521, Japan, [3]Orthopedic Surgery, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto, Nagano 390-8621, Japan

* Corresponding author (present address);

Division of Molecular Chemistry, Graduate School of Engineering, Hokkaido University, Sapporo, 060-8628, Japan.

Tel +81-11-706-6567, Fax +81-11-706-6567

E-mail takagi-m@eng.hokudai.ac.jp

Abstract

Effects of subcultivation of human bone marrow mesenchymal stem cells on their capacities for chondrogenesis and supporting hematopoiesis, and telomea length were investigated. Mesenchymal stem cells were isolated from human bone marrow aspirates and subcultivated several times at 37℃ under a 5% CO_2 atmosphere employing DMEM medium containing 10% FCS up to the 20th population doubling level (PDL). The ratio of $CD45^-$ $CD105^+$ cells among these cells slightly increased as PDL increased. However, there was no marked change in the chondrogenic capacity of these cells, which was confirmed by expression assay of aggrecan mRNA and Safranin O staining after pellet cell cultivation. The change in capacity to support hematopoiesis of cord blood cells was not observed among cells with various PDLs. On the other hand, telomere length markedly decreased as PDL increased at a higher rate than that at which telomere length of primary mesenchymal stem cells decreased as the age of donor increased.

Key words: mesenchymal stem cell, subcultivation, population doubling level, chondrogenesis, telomere, hematopoiesis

Running title: Subcultivation of Mesenchymal Stem Cells

Introduction

Mesenchymal stem cells (MSCs) in an adult bone marrow have been shown to give rise to multiple mesodermal tissue types, including bone and cartilage (Caplan, 1991), tendon (Caplan, 1993), muscle (Wakitani, 1995), and fat cells (Dennis, 1996). MSCs have an adherent, fibroblast-like morphology, and have surface antigens characterized as $CD45^-$, which is a representative marker of hematopoietic cells, and $CD105^+$, which is the TGF-β receptor endoglin (Pittenger, 1999; Barry, 1999). These stem cells are isolated from bone marrow aspirates and, because of their multilineage potential, present exciting opportunities for cell-based therapeutic applications. Therapeutic modalities have been described for the use of MSCs in cartilage (Wakitani, 1994), bone (Bruder, 1998), and tendon (Young, 1998) regeneration.

The bone marrow microenvironment containing stromal cells and an extracellular matrix shares an important role in the hematopoietic system such as self-renewal of hematopoietic stem cells and their differentiation to mature blood cells through progenitor cells (Dorshkind, 1990; Verfaillie, 1994). Stromal cells are considered to regulate the proliferation and differentiation of hematopoietic cells by secretion of cytokines and/or direct contact with hematopoietic cells (Broxmeyer, 1986; Dexter, 1987). There were several reports

that hematopoietic progenitors are expanded *ex vivo* by coculture with bone marrow stromal cells for application to bone marrow transplantation. (Kohler, 1999; Tsuji, 1999; Yamaguchi, 2001) It was reported that the bone marrow MSC fraction contains stromal cells. (Majumdar, 1998; Majumdar, 2000)

MSCs should be expanded *in vitro* after isolation from bone marrow for utilization in regenerative medicines, because the number of MSCs isolated from one adult bone marrow aspirate is too small (e.g., 2,000 cells (Bruder, 1997)) to effect tissue regeneration. Human bone marrow MSCs can be passaged in a medium containing fetal calf serum (FCS) up to the 38th population doubling level (PDL) while still maintaining their osteogenic potential (Bruder, 1997). On the other hand, it was reported that MSCs lose the capacities for adipogenesis and chondrogenesis after proliferation to 19 and 22 PDL, respectively. (Banfi, 2000) Moreover, telomere length generally decreases as cells proliferate and this can be a mechanism underlying carcinogenesis. (Counter, 1992; Rudolph, 1999) A proportional correlation between telomere length and life span of cells was reported. (Allsopp, 1992)

In this study, influences of subcultivation of human bone marrow MSCs on their surface antigen, capacities for chondrogenesis and supporting hematopoiesis, and telomere length were investigated.

Materials and methods

MSC preparation and subcultivation

Bone marrow was obtained from human donors (Table 1). All subjects enrolled in this study gave their informed consent, which was approved by our institutional committee on human research, as required by the study protocol. Approximately 10 ml of unfractionated bone marrow was obtained by routine iliac crest aspiration. The bone marrow aspirate was diluted with a growth medium, plated in the dish (55 cm^2, Corning, Tokyo) to a concentration of 6.0×10^5 nucleated cells/cm^2 and cultured at 37℃ in a humidified atmosphere containing 5% CO_2 for 19 d, during which time the medium was changed on days 1, 2, 9 and 16. When the culture reached subconfluence, the cells were detached using trypsin-EDTA (Sigma, St. Louis, MO, USA) and enumerated by the trypan blue dye-exclusion method. Some cells were stocked in liquid nitrogen and the remaining cells were subcultivated at a cell density of 1×10^4 cells/cm^2. When the culture reached near confluence, the cells were stocked and subcultivated further.

Primary human cord blood mononuclear cells

After cord blood (CB), which was obtained from normal full-term deliveries, was mixed with 10% aliquot of citrate solution (ACD-A, Terumo, Tokyo, Japan) for more than 1 h at 15℃, mononuclear cells (MNCs) were collected using Ficoll-Paque and frozen until use.

Media

The growth medium was DMEM-LG (Gibco, NY, USA) supplemented with 10% FCS (Gibco), 2500 U/l penicillin, and 2.5 mg/l streptomycin.

The chondrogenesis differentiation medium was DMEM-HG (Gibco) supplemented with 10% FCS, 2500 U/l penicillin, 2.5 mg/l streptomycin, 50 μg/ml l-ascorbic acid 2-phosphate (Wako Pure Chemicals, Osaka), 100 μg/ml sodium pyruvate (Wako), and 40 μg/ml proline (Wako), 10 ng/ml transforming growth factor-β3 (TGF-β3, Peprotech), and 39 ng/ml dexamethasone (ICN Biomedicals).

A serum-free medium of X-VIVO10 (Biowhittaker, Walkersvill, USA) was employed for the cultivation of CB MNCs.

Cultivation of CB MNCs

Frozen mesenchymal cells were thawed, inoculated into 24 multi-well-plates for

adhesion culture at a density of 5×10^4 cells/cm^2 and incubated for 6 h at 37℃ in 5% CO_2. Cells were exposed to 1,500 cGy of Cs^{137} γ ray irradiation using a Gamma-cell 40 irradiator (MDS Nordian, Ottawa, Canada) and washed with PBS. Frozen CB MNCs were thawed, inoculated into the 24 multi-well-plates containing mesenchymal cells mentioned above at a density of 5×10^5 cells/ml (culture volume; 1 ml) and cocultured for 7 days at 37℃ in 5% CO_2. Cocultivated mesenchymal and CB hematopoietic cells, which were harvested by trypsinization, were enumerated by the trypan blue method and used for further assay of colony forming units.

Colony-forming unit (CFU) assay

The suspended hematopoietic cells (3×10^4 cells) were washed and resuspended in 2.5 ml of the assay medium (Methocult GF 4434V, Stem Cell Technologies) composed of Iscove's modified Dulbecco's medium, 1% methylcellulose, 10^{-4} M 2-mercaptoethanol, 2 mM *L*-glutamine, 30% FBS, 1% BSA, 3 U/ml recombinant human erythropoietin, 50 ng/ml recombinant human SCF, 10 ng/ml recombinant human granulocyte macrophage – colony-stimulating factor, 10 ng/ml recombinant human interleukin 3, and 20 ng/ml recombinant human granulocyte – colony-stimulating factor. Two multiwell plates (35 mm ϕ, Corning), each containing 1 ml of the same cell suspension, and one multiwell plate containing

only 3 ml of sterilized water were placed in a dish (100 mmϕ, Corning) and incubated at 37℃ in a humidified incubator containing 5% CO_2. The numbers of CFUs of the mixture (CFU-Mix), CFUs of granulocyte macrophage (CFU-GM), and burst-forming units of erythrocyte (CFU-E) were counted under an inverted microscope on day14 and total number of CFUs was shown as the total number of progenitor cells.

Pellet cell cultivation

Frozen mesenchymal cells were thawed, inoculated into a dish for adhesion culture (55 cm^2, Corning) at a density of 5×10^4 cells/cm^2, incubated for 6 h at 37℃ in 5% CO_2, and harvested by trypsinization. High-density pellet cell cultures were initiated by centrifugation (500×g for 5 min) of 5×10^5 cells suspended in 0.5 ml of the differentiation medium in 15-ml conical tubes. The pellet cells were incubated for 2 wk at 37℃ in 5% CO_2, during which time the medium was changed weekly. After the pellet was hydrolyzed at 37℃ for 40 min with 5 g/l trypsin (Sigma), 5 g/l type 2 collagenase (Worthington Biochemistry), and 5 g/l type 1 collagenase (Wako, Osaka, Japan), cell concentration was determined by the trypan blue method.

Flow cytometry analysis

Frozen mesenchymal cells were thawed, inoculated into a dish for adhesion culture (55 cm^2, Corning) at a density of 5×10^4 cells/cm^2, incubated for 6 h at 37℃ in 5% CO_2, and harvested by trypsinization. Cells were stained with mouse IgG anti-human CD105 (Beckman Coulter) and phycoerythrin (PE)-conjugated anti-murine IgG (Beckman Coulter). Thereafter, cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-human CD45 antibodies (Beckman Coulter), and analyzed using a flow cytometer (EPICS XL, Beckman Coulter) equipped with an argon laser (488 nm).

Staining

The pellet was rinsed twice with PBS, fixed in 20% formalin, dehydrated through a graded series of ethanol, infiltrated with isoamyl alcohol, and embedded in paraffin. Sections of 3 μm thickness were cut across the center of the pellet and gel, and were stained with 1% Safranin O in 1% sodium borate

RNA preparation and RT-PCR

Total RNA was prepared from cells in a pellet (n=3 at each time point) using an RNeasy mini kit (Qiagen, Maryland, USA). Dnase-treated RNA was used to produce cDNA employing Omniscript and Sensiscript RT kits (Qiagen) and Gene

Amp PCR System 9700 (Applied Biosystems, USA, Foster City). PCR amplification of the cDNA was performed employing a HotStar Tag Master Mix kit (Qiagen) and ABI PRISM 7700 (Applied Biosystems) using actin as the inner standard (NM 001101, Applied Biosystems). The sequences of primers and probes are listed in Table 2. The cDNA prepared with RNA isolated from primary human chondrocytes from an articular cartilage was also employed for the PCR analysis. The ratio of mRNA expression level in cultured cells to that in primary cells was calculated to obtain the degree of expression of aggrecan.

Analysis of telomere length

DNA was prepared from MSCs (n=3 at each PDL) using the DNeasy tissue kit (Qiagen), and telomere length was analyzed by TRF (terminal restriction fragment) assay employing a Telo TAGGG Telomere Length Assay kit (Roche Molecular Biochemicals). Briefly, DNA was digested with restriction enzymes, *Rsa I* and *Hinf I*, and carefully quantified by a fluorometric method. A portion (2 μg) was loaded onto a 0.8% agarose gel and resolved by electrophoresis. DNA was transferred to a nylon membrane (Roche), hybridized with a digoxigenine-labeled telomere-specific probe and quantified using Typhoon 9210 (Molecular Dynamics) and Image Quant (Amersham Biosciense).

Statistical analysis

All experiments were performed more than two times and similar results were confirmed. Significant differences ($P < 0.05$) were established using Student's t-test.

Results

Proliferation of adherent cells in subcultivation

Adherent cells in bone marrow aspirates from donors A to F in Table 1 were isolated and subcultivated. Population doubling level (PDL) was calculated using cell density change during each subcultivation on the assumption that the content of adherent cells among bone marrow cells was 0.1%.[24] Cells proliferated stably until 8 to 20 PDL. (Figure. 1)

Content of $CD45^-$ $CD105^+$ cells among adherent cells

Surface antigen was analyzed using a flowcytometer for cells harvested in the subcultivations. The percentage of $CD45^-$ $CD105^+$ cells among adherent cells was maintained at higher than 80% except for cells at 5 PDL from donor B. (Figure. 2) The percentage of $CD45^-$ $CD105^+$ cells tended to increase slightly as

PDL increased.

Chondrogenesis capacity of adherent cells from bone marrow

After pellet cell culture was performed using adherent cells harvested from the subcultivations, aggrecan mRNA expression level in cells in the pellets was determined (Figure. 3) and sections of pellets were stained with Safranin O (Figure. 4). The aggrecan mRNA expression level was around 0.4% except for that of cells from donor B. There was no marked change in the expression level due to subcultivation. Cells could form the pellet and those pellets could be stained well with Safranin O independent from PDL ranging from 4.9 to 14.5. (Figure. 4)

Capacity for supporting hematopoiesis of adherent cells from bone marrow

The coculture of cord blood hematopoietic cells and adherent cells from bone marrow was performed and hematopoietic progenitor cell concentration was determined by CFU assay. (Figure. 5) Progenitor concentration after coculture with bone marrow adherent cells did not increase with an increase in PDL except for that corresponding to the PDL increase from 12.6 to 14.5 of adherent cells from donor A.

Analysis of telomere length of adherent cells from bone marrow

TRF assay was performed for adherent cells harvested after the incubation of bone marrow aspirates of all donors presented in Table 1, except for donor C, approximately for 20 days (Figure. 6) and for adherent cells after subcultivations of bone marrow cells from donor D, E and F (Figure. 7). The TRF length of adherent cells after the incubation of bone marrow aspirates for approximately 20 days tended to decrease as the age of donor increased and the rate of decrease was approximately –26 bp/y. (Figure. 6) On the other hand, the TRF length of adherent cells apparently decreased as PDL increased for all donors tested and the rate of decrease was –38 to –120 bp/PDL. (Figure. 7)

Discussion

It was reported that 1×10^7 nucleated cells following the density-gradient separation of a bone marrow aspirate contained 2,200 MSCs (Bruder, 1997) and the content of MSCs among the nucleated cells was approximately 0.2%. The content of $CD105^+$ and $CD45^-$ cells among nucleated cells in bone marrow aspirates determined using a flowcytometer was less than 0.1% in our experiments. (Takagi, 2003) Therefore, the PDL of adherent cells harvested from culture of bone marrow aspirates was calculated on the assumption that the

content of MSCs among nucleated cells in bone marrow aspirate is 0.1%.

Generally, 10 ml of bone marrow aspirate contains approximately 1×10^9 nucleated cells and 1×10^5 MSCs even when the content of MSCs among nucleated cells is as low as 0.1%. (Pittenger, 1999) On the other hand, cartilage (5 cm in length, 2 cm in width, 4 mm in thickness, cell density of 5×10^6 cells/ml) contains 2×10^7 chondrocytes. Thus, MSCs should be expanded at least 200-fold for regenerative medicine. This increase in cell number corresponds to the increase of 8 PDL and adherent cells derived from bone marrow aspirates can reach a PDL higher than 8 as obtained in this study (Figure. 1).

The average doubling time of adherent cells from bone marrow aspirates, which is shown as the inverse of slope in Figure. 1, varied from 91 to 194 h. However, there was no apparent correlation between the average doubling time and the characteristics of donors such as age and gender (data not shown).

The content of SH-2-positive ($CD105^+$) $CD45^-$ cells among adherent cells prepared from bone marrow aspirates was reported to be approximately 98%. (Majumdar, 1998) In our previous report, high content of $CD105^+$ $CD45^-$ cells corresponded to high chondrogenesis capacity (Takagi, 2003). So, CD105 was employed as a marker of MSCs while MSCs generally express other markers in addition to CD105. The content of $CD105^+$ $CD45^-$ cells among nucleated adherent cells harvested after the incubation of bone marrow cells was

approximately 90% as shown in Figure. 2 and it was slightly lesser than that reported previously. However, almost all adherent cells harvested from bone marrow aspirates were proved to be mesenchymal stem cells and there was no decrease in the content even after the subcultivation to 19 PDL.

The chondrogenic capacity of adherent cells after subcultivations was investigated by the assay of aggrecan mRNA expression and Safranin O staining after pellet culture. The expression level was less than 1% compared with primary human cartilage chondrocytes (Figure. 3) and this may partly be due to the short duration (2 wk) of pellet culture. However, these chondrogenesis capacities might not too low, because the darkness of stained pellet sections was comparable to those of Safranin O stained pellet sections composed of fresh primary chondrocyte cells. (data not shown) There was no marked decrease in the expression level as PDL increased to 18. All cells shown in Figure. 3 could form the pellets although nucleated cells in bone marrow aspirates could not form any pellet. (data not shown) Unstained areas in the center of a pellet section as shown in Figure. 4 were often observed in pellet culture. All other parts of the pellet section could be stained well independent of donors and PDL. These results strongly suggested that adherent cells from bone marrow aspirates can maintain their chondrogenic capacity during subcultivations at least up to 15 PDL.

The cocultivation of cord blood hematopoietic cells and adherent cells from bone marrow aspirates was performed in order to study the influence of PDL on the capacity for supporting hematopoiesis. (Figure. 5) Hematopoietic progenitor concentrations were well maintained from 0.3 to 1.0×10^3 cells/ml. Thus, this result suggests that subcultivation up to 15 PDL does not affect the capacity for supporting hematopoiesis of adherent cells from bone marrow aspirates.

In order to study the effect of subcultivation on the telomere length of adherent cells from bone marrow, TRF assay was performed for adherent cells harvested from bone marrow aspirates after 19 days cultivation (Figure. 6) and for those harvested after several subcultivations (Figure. 7). Subcultivated bone marrow adherent cells derived from donors of D, E, and F were employed in Figure. 7 because those cells were subcultivated to higher PDL compared with cells from other donors. Figure 6 shows that the telomere length was about 8 to 10 kbp and decreased as the age of donor increased. The decrease rate was approximately -26 bp/y. Lustiga reported that the telomere length of human cells was approximately 10 kbp. (Lustiga, 1990) It was also reported that telomere length becomes shorter as age increases (Harley, 1990) and the decrease rate varies from -15 to -100 bp/y depending on the tissue types (Takubo, 2002). Our results shown in Figure. 6 agree with those reported. TRF assay was also performed for adherent cells harvested after several subcultivations and telomere

length was proved to be shorter as PDL increased (Figure. 7) The rates of decrease in telomere length with subcultivation were from -38 to -120 bp/PDL. Harley also reported that the rate of decrease in the telomere length of fibroblasts *in vitro* was approximately 50 bp/PDL. (Harley, 1990) The rate of decrease in telomere length by 1 PDL was proved to be equal to that by 1.5 (38/26) to 4.5 (120/26) years. There was a report that bone marrow transplantation from donors of advanced age may be dangerous because of the decreased telomere length. (Akiyama, 1998a; Akiyama, 1998b) Furthermore, *in vitro* proliferation life was proportional to telomere length and a minimum telomere length of 5 kbp was necessary for *in vivo* proliferation. (Allsopp, 1992)

Conclusions

The decrease in telomere length should be a focus of much attention before clinical application of *in vitro* expanded mesenchymal stem cells, while there may be no marked changes in surface antigens and capacities for chondrogenesis and supporting hematopoiesis after several subcultivations.

References

Akiyama M, Hoshi Y, Sakurai S, Yamada H, Yamada O and Mizoguchi H (1998a)

Changes of telomere length in children after hematopoietic stem cell transplantation. Bone Marrow Transplantation 21(2): 167-171.

Akiyama M, Uchiyama H, Hoshi Y, Yano S, Asai O, Kuraishi Y, Yamada O, Mizoguchi H and Yamada H (1998b) Changes of telomere length after hematopoietic stem cell transplantation. Experimental Hematology 26(8): 359-365.

Allsopp RC, Vaziri H, Patterson C, Goldstein S, Younglai EV, Futcher AB, Greider CW and Harley CB (1992) Telomere length predicts replicative capacity of human fibroblasts. PNAS USA 89(21): 10114-10118.

Banfi A, Muraglia A, Dozin B, Mastrogiacomo M, Cancedda R and Quarto R (2000) Proliferation kinetics and differentiation potential of *ex vivo* expanded human bone marrow stromal cells: Implications for their use in cell therapy. Exp Hematol 28: 707-715.

Barry FP, Boynton RE, Haynesworth S, Murphy JM and Zaia J (1999) The monoclonal antibody SH-2, raised against human mesenchymal stem cells, recognizes an epitope on endoglin (CD105). Biochem Biophy Res Commun 265: 134-139.

Broxmeyer HE (1986) Biomolecule-cell interactions and the regulation of myelopoiesis. Int J Cell Cloning 4: 378-405.

Bruder SP, Jaiswal N and Haynesworth SE (1997) Growth kinetics, self-renewal,

and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. J Cell Biochem 64: 278-294.

Bruder SP, Kurth AA, Shea M, Hayes WC, Jaiswal N and Kadiyala S (1998) Bone regeneration by implantation of purified, culture-expanded human mesenchymal stem cells. J Orthop Res 16: 155-162.

Caplan AI (1991) Mesenchymal stem cells. J Orthop Res 9: 641-650.

Caplan AI, Fink DJ, Goto T, Linton AE, Young RG, Wakitani S, Goldberg VM and Haynesworth SE (1993) Mesenchymal stem cells and tissue repair. In Jackson, D.W. (ed.): The anterior cruciate ligament: Current and future concepts. New York: Raven Press, Ltd., pp 405-417.

Counter CM, Avilion AA, LeFeuvre CE, Stewart NG, Greider CW, Harley CB and Bacchetti S (1992) Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. Embo J 11: 1921-1929.

Dennis JE and Caplan AI (1996) Differentiation potential of conditionally immortalized mesenchymal progenitor cells from adult marrow of a H-2K^{b}-tsA58 transgenic mouse. J Cell Physiol 167: 523-538.

Dexter TM and Spooncer E (1987) Growth and differentiation in the hemopoietic system. Annu Rev Cell Biol 3: 423-441.

Dorshkind K (1990) Regulation of hemopoiesis by bone marrow stromal cells and their products. Annu Rev Immunol 8: 111-137.

Harley CB, Futcher AB and Greider CW (1990) Telomeres shorten during ageing of human fibroblasts. Nature 345: 458-460.

Kohler T, Plettig R, Wetzstein W, Schaffer B, Ordemann R, Nagels HO, Ehninger G and Bornhauser M (1999) Defining optimum conditions for the *ex vivo* expansion of human umbilical cord blood cells. Influences of progenitor enrichment, interference with feeder layers, early-acting cytokines and agitation of culture vessels. Stem cells. 17(1): 19-24.

Lustiga J, Kurtz S and Shore D (1990) Involvement of the silencer and UAS binding-protein RAPI in regulation of telomere length. Science 250(4980): 549-553.

Majumdar MK, Thiede MA, Mosca JD, Moorman M and Gerson SL (1998) Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells. J Cell Phys 176: 57-66.

Majumdar MK, Thiede MA, Haynesworth SE, Bruder SP and Gerson SL (2000) Human marrow-derived mesenchymal stem cells (MSCs) express hematopoietic cytokines and support long-term hematopoiesis when differentiated toward stromal and osteogenic lineage. J Hematother Stem Cell Reserch 9: 841-848.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S and Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science 28: 143-147.

Rudolph KL, Chang S, Lee HW, Blasco M, Gottlieb GJ, Greider C, DePinho RA (1999) Longevity, stress response, and cancer in aging telomerase-deficient mice. Cell 96: 701-712.

Takagi M, Nakamura T, Matsuda C, Hattori T, Wakitani S and Yoshida T (2004) *In vitro* proliferation of human bone marrow mesenchymal stem cells employing donor serum and basic fibroblast growth factor. Cytotechnology, 43, 89-96 (2003).

Takubo K, Izumiyama-Shimomura N, Honma N, Sawabe M, Arai T, Kato M, Oshimura M and Nakamura KI (2002) Telomere lengths are characteristic in each human individual. Experimental Gerontology 37(4): 523-531.

Tsuji T, Nishimura-Morita Y and Watanabe Y (1999) A murine stromal cell line promotes the expansion of $CD34^{high+}$-primitive progenitor cells isolated from human umbilical cord blood in combination with human cytokines. Growth Factors 16: 225-240.

Verfaillie C, Hurley R, Bhatia R (1994) Role of bone marrow matrix in normal and abnormal hematopoiesis. Crit Rev Oncol Hematol 16: 201-224.

Wakitani S, Goto T, Pineda SJ, Young RG, Mansour JM, Caplan AI and Goldberg

VM (1994) Mesenchymal cell-based repair of large full-thickness defects of articular-cartilage. J Bone Joint Surg Am 76: 579-592.

Wakitani S, Saito T and Caplan AI (1995) Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. Muscle Nerve 18: 1417-1426.

Yamaguchi M, Hirayama F, Kanai M, Sato N, Fukazawa K, Yamashita K, Sawada K, Koike K, Kuwabara M, Ikeda H and Ikebuchi K (2001) Serum-free coculture system for *ex vivo* expansion of human cord blood primitive progenitors and SCID mouse-reconstituting cells using human bone marrow primary stromal cells. Exp Hematol 29: 174-182.

Young RG, Butler DL, Weber W, Caplan AI, Gordon SL and Fink DJ (1998) Use of mesenchymal stem cells in a collagen matrix for Achilles tendon repair. J Orthop Res 16: 406-413.

Table 1　Human MSC donor profile

Donor	Age	Gender
A	56	F
B	60	F
C	40	F
D	46	F
E	58	F
F	58	F
G	52	M
H	73	F
I	27	M
J	25	M
K	23	F
L	13	M

Table 2　Sequences used in PCR

Human aggrecan	
Sense	5' - AGTCCTCAAGCCTCCTGTACTCA - 3'
Antisense	5' - GCAGTTGATTCTGATTCACGTTTC - 3'
Probe	5' - ATGCTTCCATCCCAGCTTCTCCGG - 3'

Figure. 1. Proliferation of adherent cells from bone marrow.　Adherent cells in bone marrow aspirates from donors A to F shown in Table 1 were isolated and subcultivated.　Population doubling level (PDL) was calculated using cell density change during each subcultivation on the assumption that the content of adherent cells among bone marrow cells was 0.1%.　Donors A(○), B(△), C(◇), D(◆), E(■), and F(●).　Each point shows the average of duplicate.

Figure. 2. Percentage of $CD45^-$ $CD105^+$ cells among adherent cells from bone

marrow. Surface antigen was analyzed using a flowcytometer for cells harvested from subcultivations shown in Figure. 1. Symbols are the same as those in Figure. 1. Each point shows the average of duplicate for donors A, B, and C, and triplicate for donors D, E, and F. The bar indicates standard deviation.

Figure. 3. Expression of aggrecan mRNA in pellet cell culture of adherent cells from bone marrow. The expression of aggrecan mRNA in cells in the pellets was determined after pellet cell culture was performed using adherent cells harvested from subcultivations. Symbols are the same as those in Figure. 1. Each point shows the average of duplicate for donors A, B, and C, and triplicate for donors D, E, and F. The bar indicates standard deviation.

Figure. 4. Staining of pellets with Safranin O. Pellet sections were stained with Safranin O after pellet cell culture was performed using adherent cells harvested from subcultivations of bone marrow cells from donors A, B, and C.

Figure. 5. Hematopoietic progenitor concentration after coculture with adherent cells from bone marrow. The hematopoietic progenitor concentration was determined after the coculture of cord blood hematopoietic cells and adherent cells harvested from subcultivations. Symbols are the same as those in Figure. 1. Each point shows the average of triplicate and the bar indicates standard deviation.

Figure. 6. Influence of donors age on TRF length of bone marrow adherent cells. TRF assay was performed for adherent cells harvested after incubation of bone marrow aspirates from all donors shown in Table 1, except for donor C, approximately for 20 days.